CHAPTER 14

Klun & Debboun Modules: Uses and Data Analysis

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CONTENTS

IN VIVO K & D MODULE BIOASSAY

The first Klun and Debboun module (K & D module) was designed for in vivo experimental use on humans[1] to evaluate the mosquito-feeding deterrent efficacy of chemicals that were toxicologically safe for application on the skin of humans. Impetus for development of this bioassay system resulted from problems with experimental techniques developed and used on humans between 1983 and 1992. These methods often required lengthy periods for observation and reducing the number of replications. The physical designs of the apparatus, where mosquitoes comingled in a common area and might have switched between feeding areas, made the data multinomial and induced correlations, making them more difficult to correctly analyze.

Details of the K & D module are shown in Figure 14.1. The module was made of acrylic plastic and with six 100-cm^3 individual cells. Each cell had a stopper-access hole for transfer of mosquitoes into the cell and a sliding bottom door (Figure 14.1, F). The module was designed to be used on a human thigh. It was designed concave to conform to the curvature of a human thigh (Figure 14.1, End view). A separate length of acrylic plastic, identical to the module's base with six rectangular

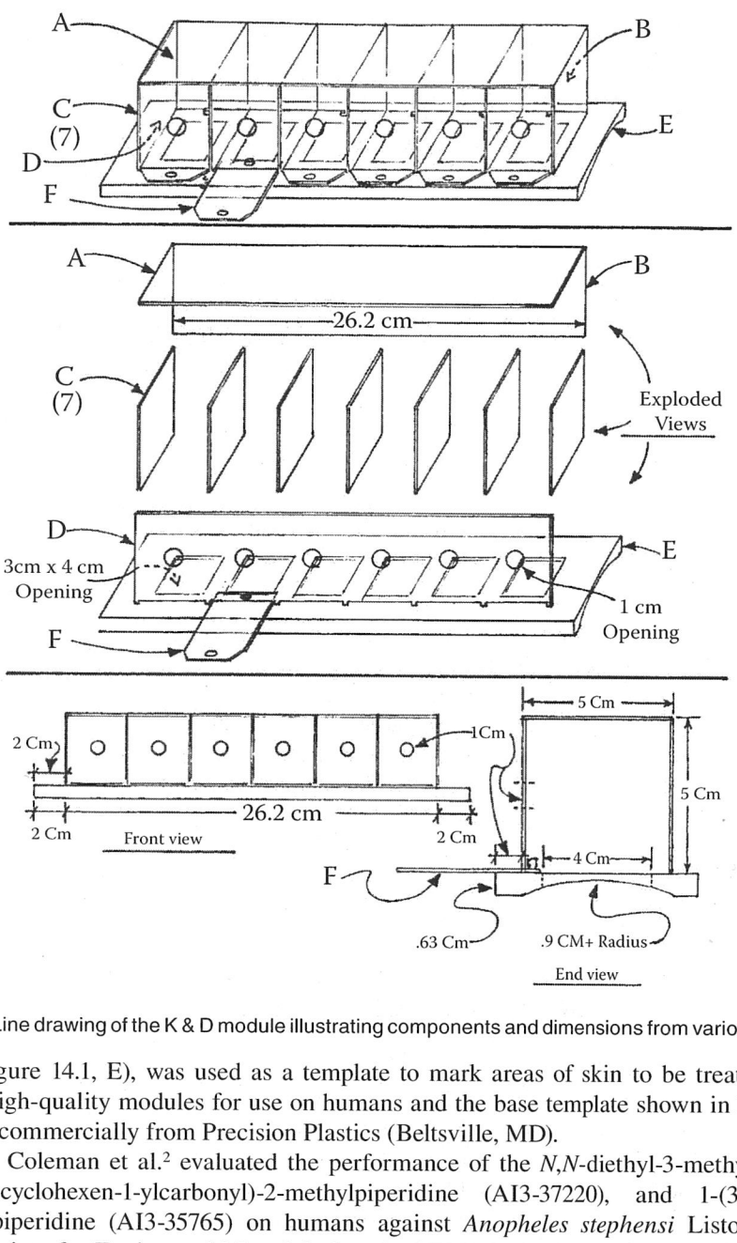

Figure 14.1 Line drawing of the K & D module illustrating components and dimensions from various viewpoints.

openings (Figure 14.1, E), was used as a template to mark areas of skin to be treated with test chemicals. High-quality modules for use on humans and the base template shown in Figure 14.2a are available commercially from Precision Plastics (Beltsville, MD).

Early on, Coleman et al.[2] evaluated the performance of the *N,N*-diethyl-3-methylbenzamide (deet), 1-(3-cyclohexen-1-ylcarbonyl)-2-methylpiperidine (AI3-37220), and 1-(3-cyclohen-1-ylcarbonyl) piperidine (AI3-35765) on humans against *Anopheles stephensi* Liston using the American Society for Testing and Materials Standard E951-8 plastic cage bioassay.[3,4] The plastic cage was open and rectangular (18 cm × 5 cm × 4 cm = 360 cm^3) with a screened top and five circular holes (29 mm diameter) in the floor, and a slide that permitted opening and closing of the holes (Figure 14.2b). In practice, a template matching the floor openings was used to mark and randomly treat circular areas on a volunteer's forearm. One area served as control and the remaining four were randomly treated with different doses of repellent chemical. The plastic cage, filled with 20 mated female mosquitoes (mosquito density equivalent to 0.05 female/cm^3), was secured over the treated forearm skin areas, and the floor slide was pulled out to expose all mosquitoes to all five skin treatments. The number of insects biting on each treatment site within the plastic cage was

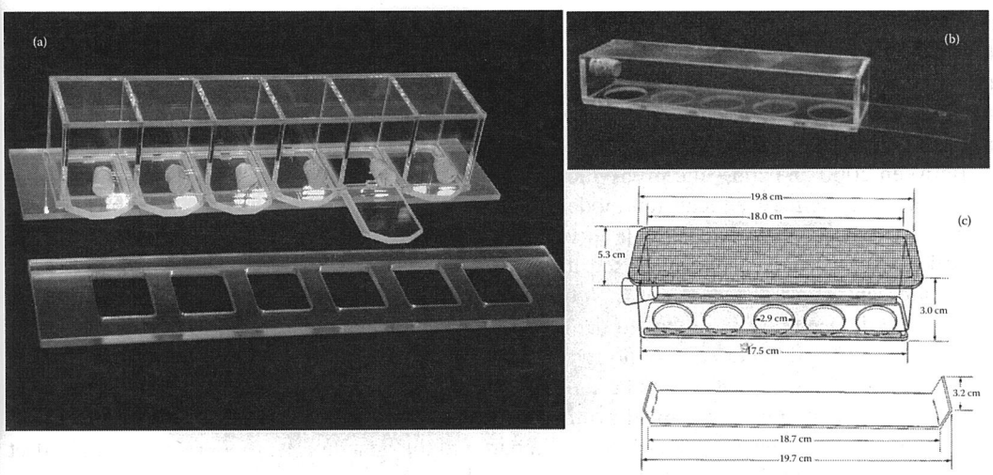

Figure 14. 2 (a) K & D module made of acrylic plastic. Template for marking of volunteer's thigh areas for treatments is shown in front of the module. Modules constructed with acrylic plastic are durable and washable in automatic washers. The module and template can be obtained from Precision Plastics (Beltsville, MD). (b) American Society for Testing and Materials Standard E951-8 plastic cage design. (c) Modified E951-8 cage design (from Gupta et al.[5]) for autoclave cleaning, and providing dimensions (same as for the E951-8 plastic cage design).

recorded during each minute of a 5-minute test period. This design was modified by Gupta et al.[5] so that it could be easily autoclaved between uses, though proportions were not changed (Figure 14.2c, from Gupta et al.[5]).

In the very first test of its bioassay power, the in vivo K & D module was used to evaluate the three compounds that Coleman et al.[2] had previously tested in vivo against *Anopheles stephensi*. In the testing, each of the four module cells was charged with five mosquitoes making for a mosquito density equivalent to the density Coleman et al.[2] used in their E951-8 plastic cage tests. Notably, all K & D tests conducted after 2000 used an insect density of five mosquitoes/cell. Data obtained with the in vivo K & D module showed that deet and AI3-37220 performed equally well against the mosquitoes and that AI3-35765 was the least effective. Data gathered by Coleman et al.[2] supported the same conclusion, but they required 36 replicates for their evaluation of the 3 compounds. In contrast, the K & D module provided the same quantitative results with 18 replicates. The improved bioassay efficiency over the open plastic cage (which simultaneously exposed all mosquitoes to all treatments) was attributed to the K & D module design that isolated the mosquitoes and dosage treatments in a replicate, eliminating the need for each mosquito to assess all treatments or doses available in the open cage, and reducing sampling variability. Isolated cells of the K & D module not only eliminated the need for mosquitoes to choose among treatments or doses but also permitted designs that better isolate the sources of variation. For example, one can simultaneously evaluate several species against an individual repellent at a single dose or the responses of several species to several candidate-repellent chemicals or doses. The K & D module assay system, either in vivo or in vitro, was designed to be utilized with chemicals having low vapor pressures, similar to that of deet. Use of candidate chemicals with higher vapor pressures could result in leakage between cells.

Rutledge and Gupta[6] referred to the isolated cells of the K & D module design as the "no-choice test module" and advocated that test systems should be designed, or redesigned, to function in the no-choice mode to provide results with less variability. Furthermore, in contrast with the Standard E951-8 plastic cage, which was positioned on the forearm of a volunteer and permitted two replicates

to be tested within one volunteer, the K & D module can be used on the outer, top, and inner thigh of each leg of a volunteer, permitting six replicates to be tested in one sitting of each volunteer. This gave the K & D module more replicated units than the Standard E951-8 plastic cage, though each unit had fewer mosquitoes. In general, this is an asset: experimental design recommendations are to have smaller and more numerous blocks.[7]

Between 2000 and 2003, the in vivo K & D module was successfully used to study the influence of optically active chemicals (antipodes) against mosquito blood-feeding behavior.[8,9] The optically active compounds were available in very limited amounts (5–10 g each), but testing of these antipodes was possible because of the sensitivity of the K & D assay that required only micrograms per square centimeter skin doses of the precious compound applied to the volunteers' skin. The K & D module was used in studies to demonstrate the importance of optical forms in mosquito-repellent activity and led to the patenting of a most potent optical form of the repellent chemical (1*S*, 2*S*′)-2-methylpiperidinyl-3-cyclohexen-1-carboxamide (SS220).[10] It was then used in a study to develop an efficient organic chemical synthetic method for preparation of SS220, and to evaluate the compound's performance as a repellent compared to the benchmark-repellent compounds, deet and hydroxyethylbutyl piperidine carboxylate (Picaridin), against two species of mosquitoes that are important vectors of yellow fever, dengue, and malaria.[11] This work was part of a broader objective to develop a new, effective, and safe repellent product for use against arthropods that are disease vectors. By using the in vivo K & D module with human volunteers, it was demonstrated that the protection afforded by deet, Picaridin, and SS220 against *Aedes aegypti* (Linnaeus), *Anopheles stephensi*, and *Phlebotomus papatasi* Scopoli bites was due to repellent and deterrent effects[12] (according to the standard terms developed by Dethier et al.[13] to describe chemicals in terms of the behavioral response they evoke). Readers are encouraged to review this publication[12] in detail because the article reveals several innovative ways that the in vivo K & D module can be used to reveal the behavioral mode of action of compounds against mosquitoes and sand fly blood feeding.

Figure 14.3 depicts the step-by-step use of an in vivo K & D module on a human volunteer in a quantitative experiment designed to evaluate the dose × response of *Aedes aegypti* to SS220, deet, and Picaridin.[11] Volunteers who participated in the bioassays were not selected for participation by using any prescribed volunteer-selection characteristics. A person's willingness to participate by offering their skin for exposure to chemical treatment and insect bites was the only requirement for a volunteer's involvement in experiments. Bioassays were always conducted without any prescribed prebioassay treatment or conditioning of a volunteer's skin. We thought that such skin conditioning might bias test results. Volunteers were always required to sign a consent form and verify that they were not susceptible to allergic reactions from insect bites. Before the initiation of in vivo K & D module testing was undertaken, it was verified that all planned test procedures conformed to the established National Institute of Health guidelines for tests involving humans, and also complied with the approved protocols established by a local Human-Use Research Committee. In addition, it was solidly confirmed that the compounds applied to volunteers had abundant chemical safety databases that allowed dermal application to humans. The source and chemical purity of the compounds used were also firmly established. This quality control step is absolutely essential because the validity, integrity, and meaningfulness of bioassay results are dependent on the verified identity and purity of the chemicals being tested.

The in vivo K & D module human bioassay method could be usefully applied in final stages of product development and marketing of a chemical that has cleared Environmental Protection Agency toxicology testing requirements, and where developers might wish to validate a product's performance for protection of humans against important mosquito and sand fly vectors of human diseases before it is released for public use. Moreover, there is good published evidence[8–12] that the in vivo K & D module–based bioassay method is robust, practical, and useful in research and development of new effective repellent chemical products.

Figure 14.3 **(See color insert.)** (a) The seated volunteer uses an ink pen and an acrylic plastic template, representing the base and 3 cm × 4 cm openings of the K & D module, to mark skin areas of his thigh to be treated with 0, 3, 6, 12, 24, and 48 nmol/cm^2 skin doses of repellent chemical against *Aedes aegypti*. (b) Shows skin areas marked for treatment. Each row of six (3 cm × 4 cm) rectangular marks running down the volunteer's leg represents where a six-celled K & D module will be positioned on the volunteer's legs. Each row represents one replicate test of six repellent doses. Six treatments for each skin area in a row are randomly assigned for application to both legs of a person and yield six randomized replicates (blocks) per volunteer (in effect, a split plot design). (c) Shows the procedure for loading each of a module's six cells with five female *Aedes aegypti* from a 1 gallon screened carton holding 5- to 15-day-old male and female mosquitoes. Mosquitoes were usually maintained with sugar-water moistened cotton balls, but were provided water only 24 hours and no water for another 24 hours before being used in a bioassay. This treatment optimized the propensity of mosquitoes to feed in the bioassay. Once a set of mosquitoes have been transferred to a module, they should be utilized in the bioassay within 45 minutes to assure maximum biting propensity. (d) Shows randomized and replicated dose treatments being applied in 55 µL ethanol to marked areas of inner, top, and outer thigh skin surfaces. In applying a treatment, the solution is applied as uniformly as possible over the 3 cm × 4 cm and about 0.5 cm outside of the rectangular marking to assure that all skin surface subsequently exposed to the insects contains test chemical. Thus, the treatment solution is applied over a 4 cm × 5 cm area (20 cm^2) of skin, but the test insect is exposed only to a 3 cm × 4 cm area of skin. As a rule for general screening tests, chemicals being tested on human skin should be applied at a rate of 24–50 nmol/cm^2 skin. In this dose range, deet suppresses mosquito biting by about 80% compared to untreated skin.[11] (e) Sliding doors of each cell of the module are opened to expose the five mosquitoes in each cell to skin below for 2 minutes. (f) Mosquitoes are shown feeding on a control area of untreated skin after a 2-minute exposure to the skin. The number of mosquitoes probing the skin surface and engorging in each cell of the K & D module is recorded. Inspection of the figure shows that four of five mosquitoes are on the skin probing and engorging. The fifth mosquito is sitting on the plastic of the cell interior. The number of insects biting (in this case, four) in a cell is recorded and then its door is then slowly closed causing the mosquitoes to leave the skin surface and fly up into the closing cell.

IN VITRO K & D MODULE BIOASSAY SYSTEM

Despite the demonstrated effectiveness of the in vivo K & D bioassay in repellent research, limitation of its use to compounds known to be toxicologically safe constituted a severe restriction to chemical screening programs and discovery of new and effective repellents. This restriction provided impetus for development of an in vitro K & D bioassay system.[14] Our objective was to design a new bioassay system that would be equivalent to conducting assays using humans, but without the use of volunteers. Composition and organization of the system is shown diagrammatically in Figure 14.4. Figure 14.5 shows a picture of the system components.

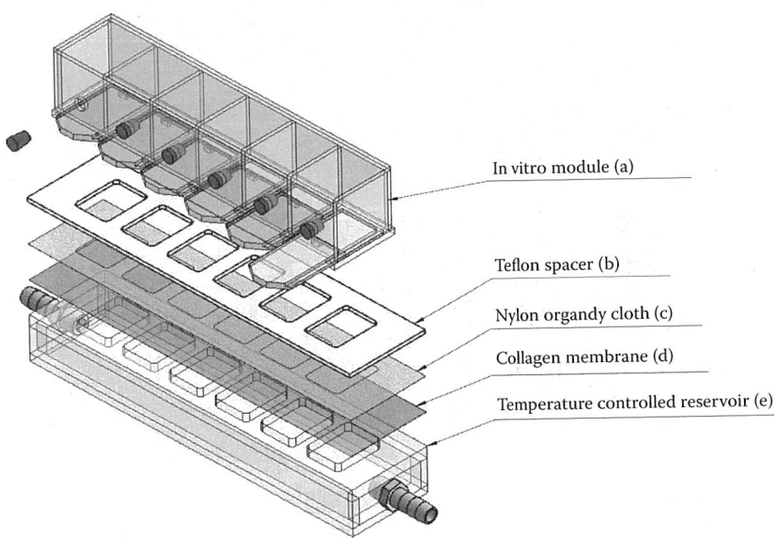

Figure 14.4 The in vitro module (a) for mosquito containment shown at the top of the diagram is identical to the in vivo module used with human volunteers (Figure 14.1) except the base of the module is flat, and not curved as was the case for the in vivo module. Component (b) is a spacer made of Teflon® that is identical to the base of the in vitro module having rectangular holes similar to the floor openings of (a). The purpose of the Teflon spacer is to prevent the module from contacting and becoming contaminated by test chemicals that are applied to organdy cloth (c). The cloth is marked with rectangular pen markings that complement the door openings of (a). Test chemicals in ethanol solution are applied to the marked rectangular areas on the cloth. The treated cloth is placed over an Edicol collagen membrane (d) that covers the six rectangular wells of a temperature-controlled (38°C) reservoir (e). The wells are filled with an aqueous preparation for mosquitoes to feed on and engorge. By design, the combined components (d) and (e) of the system represent a pseudo-human host for mosquitoes. They provide "pseudo-skin" to bite through, warmth (38°C), and a liquid below the membrane (pseudo-blood) to engorge upon. In our early use of the in vitro system, reservoir cells were filled with outdated human red blood cells suspended in anticoagulant-preservative (CPDA-1) obtained from a local blood bank supplemented with adenosine triphosphate (ATP) to cause biting mosquitoes to engorge.[15] Use of the blood cells raised significant health and logistic issues for conducting the bioassays. In 2008, we determined that citrate, phosphate, dextrose, and adenine (CPDA-1) and ATP (10^{-3} M) alone would stimulate mosquitoes to engorge.[16] This discovery led to elimination of human red blood cells from the bioassay, and enhanced the efficiency and biological safety of the assay. CPDA-1 aqueous solution, used as a mosquito-ingestion stimulus, was prepared by dissolving 3.33 g sodium citrate, 0.376 g citric acid, 0.28 g monobasic sodium phosphate, 4.02 g dextrose, and 0.035 g adenine in 126 mL water. This corrects a mangled recipe for CPDA-1 published in 2008[16] (printed as: 33.3 g sodium citrate, 0.376 g monobasic sodium phosphate, 4.02 g dextrose, and 0.35 g adenine in 63 mL water). If one has a 126 mL solution of CPDA-1 as presented here, and wishes to prepare a 10^{-3} M ATP solution from it, one would add 69.44 mg ATP (MW 551.14) to the 126 mL CPDA-1 solution. A convenient online molarity calculator for any given volume of solvent is available at http://www.physiologyweb.com/calculators/molar_solution_concentration_calculator.html.

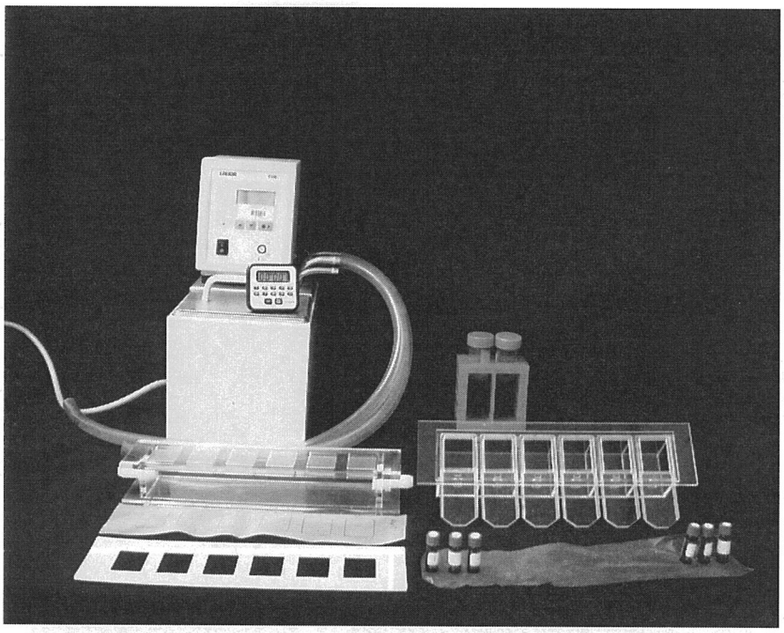

Figure 14.5 Picture of components used in the in vitro K & D bioassay. The picture shows a minute timer sitting on a water bath heater and cycling pump (38°C), and tygon tubing for connection to the six-well reservoir. Below the reservoir is a length of pen-marked nylon organdy cloth to which test chemicals will be applied. Below the cloth is the Teflon separator that was used as a template to mark the organdy cloth. A green rack holds containers of outdated packed red blood cells supplemented with adenosine triphosphate and it sits on an in vitro K & D module. Doors of the module are shown in an open position. In front of the module are six vials containing ethanol solutions of chemical treatments to be applied to the organdy cloth. The vials are standing on length of collagen membrane used to cover filled wells of the reservoir.

Figure 14.6 depicts the step-by-step use of the in vitro K & D bioassay system in a dose × response test of the repellents SS220, deet, and Picaridin. The response of *Aedes aegypti* to the three compounds was tested at 0, 3, 6, 12, 24, and 48 nmol/cm^2 cloth. The test was conducted using the same compounds and doses as was done earlier using the in vivo K & D with humans[11] (Figure 14.3). Results of the in vitro and in vivo tests with the four repellents are shown in Figure 14.7. The overall pattern of in vivo and in vitro results shows a similar decrease in biting (increase in nonbiting mosquitoes) with increased dose for all compounds. These comparative data are unique for the field of insect-repellent science in as much as we know of no other case where such a comparison of in vivo and in vitro test results has been published. As we found some differences between the in vivo and in vitro results (better compound separation and a different efficacy ranking in the in vivo system), final conclusions about a compound's utility are best drawn using the in vivo assay system after the compounds are toxicologically cleared for application to humans.

The in vitro K & D module bioassay with mosquitoes has proven to be a useful tool for discovery and characterization of chemicals that are effective against biting flies and ticks,[17–23] and a number of compounds discovered by using this bioassay have been patented.[24]

One theoretical issue that has not been fully resolved is that some attractant compounds might also inhibit feeding, especially at higher concentrations, which could also produce dose–response curves similar to those of repellents, and may be responsible for differences seen in olfactometer (where mosquitoes have the space to fly to or to avoid the test chemicals) and module testing (where mosquitoes are in small sealed cells). For example, lemon peel was found to be repellent in module

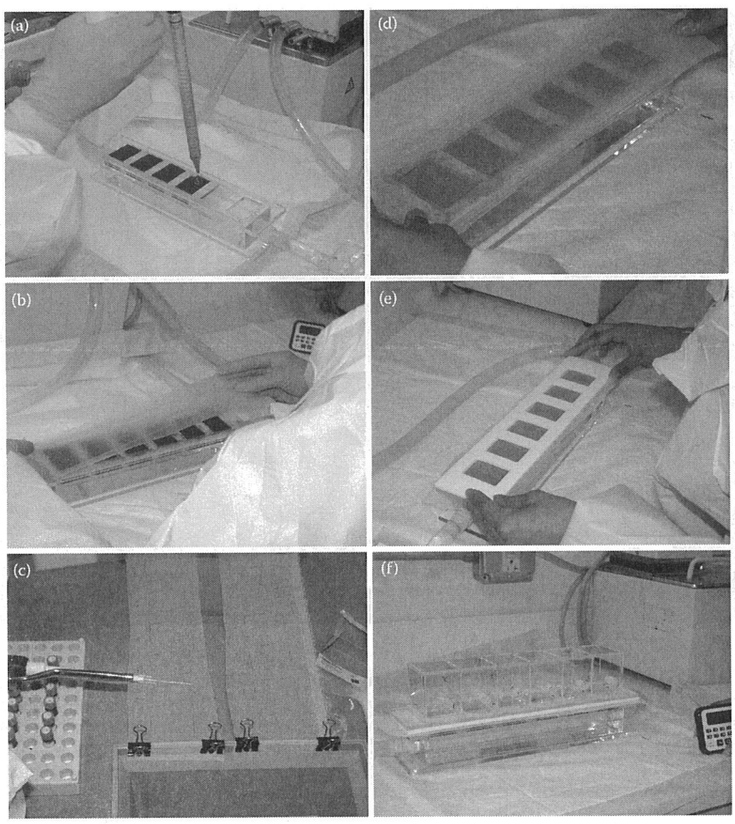

Figure 14.6 (a) Reservoir wells warmed (38°C) by water pumped from a water bath through the reservoir are filled with blood cells suspended in an aqueous solution of citrate, phosphate, dextrose, and adenine (CPDA-1) and adenosine triphosphate (ATP). Blood cells are pictured being used in this figure; however, a bioassay method developed subsequently[16] that uses an aqueous solution of CPDA-1 plus ATP alone, making use of blood cells in the bioassay obsolete. The recipe for CPDA-1 plus ATP is presented in the legend of Figure 14.4. Procedures shown in Figure 14.6 using blood cells would be the same if CPDA plus ATP are used. (b) A thin film of silicone grease is applied to edges of the reservoir and Edicol Collagen film (http://www.devro.com/our-products/edicol/edicol-a/) is secured over filled wells. (c) Five doses of compound at 3, 6, 12, 24, and 48 nmol/cm^2 cloth and ethanol alone (control) are applied uniformly to randomly marked cloth areas with a pipette. Cloth, suspended horizontally using paper clips between two trays, is treated 0.5 cm outside the 3 cm × 4 cm pen-marked area resulting in circa 20 cm^2 of treated surface. In screening tests of chemicals with unknown toxicology, treatments to cloth and the in vitro bioassays should always be made in a chemical fume hood such as a PURAIR ductless chemical fume hood (Air Science USA LLC, Fort Myers, FL). (d) Treated cloth is positioned over the Edicol collagen membrane. (e) Teflon separator is placed over the treated cloth. (f) K & D module with each cell containing five mosquitoes per cell is positioned over the Teflon separator. Mosquitoes are ready for exposure to chemical treatments. Doors of the module are opened to expose mosquitoes to treatments on the cloth surfaces, and the number of biting on the cloth surface of each treatment at the end of a 3-minute exposure is recorded. A 2-minute exposure period was routinely used in studies with in vivo human tests. The longer exposure time can be used with in vitro tests because there is no human discomfort. For high throughput screening, two reservoirs can be attached in series to a water bath with 38°C water pumped through both units. Using units in tandem increases bioassay capacity and efficiency. Empirical testing has shown that when two in vitro units are attached in series to a water bath pump, it is feasible for two technicians, working together, to screen at least 100 candidate-repellent compounds per 5-day week with 12 replicates/compound. (From Klun, J.E., Kramer, M., and Debboun, M., *J. Amer. Mosq. Control Assoc.*, 21, 64–70, 2005.)

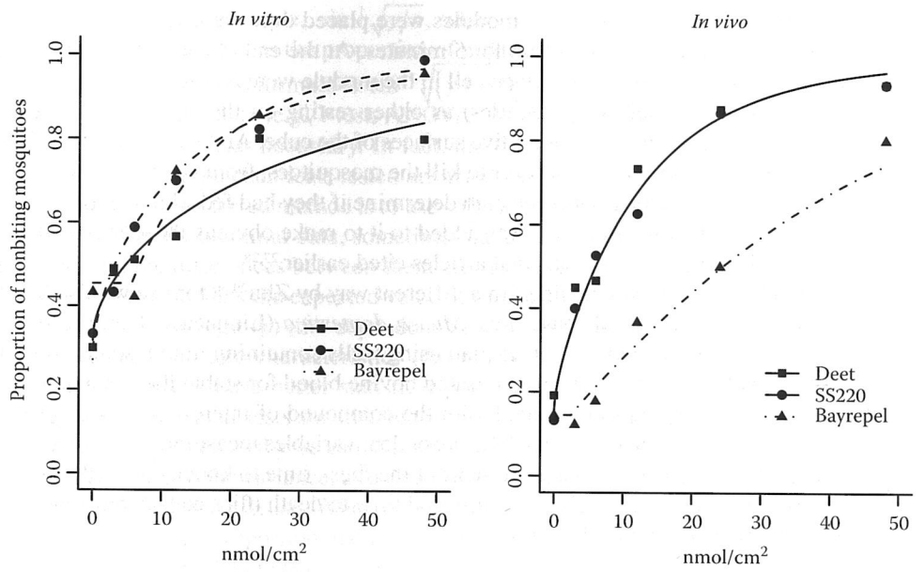

Figure 14.7 In vitro and in vivo dose–response relationships for deet, SS220, and Bayrepel for *Aedes aegypti* based on a generalized linear mixed model for the logit of the proportion of nonbiting mosquitoes. Empirical proportions of nonbiting mosquitoes calculated from the same data are also plotted with the estimated curves. Left panel: in vitro results. The dose–response lines for the three compounds are statistically indistinguishable, but best fit individual compound lines are depicted. Right panel: in vivo results. The model for deet and SS220 is logit $(p) = b_0 + b_C \sqrt{(\text{dose}_i)} + u_j$, that for Bayrepel is logit $(p) = b_0 + u_j$ for $\sqrt{(\text{dose}_i)} < 0.5$, and logit $(p) = b_0 + b_C (\sqrt{[\text{dose}_i]} - 0.5) + u_j$, otherwise; where p is the proportion of non-biting mosquitoes, i indexes the different doses, b_0 and b_C, are estimated parameters, where C indexes the three compounds, and u_j is the random effect of the jth subject, assumed to be a draw from a normal distribution with mean zero and variance estimated from the model fitting procedure (from Klun et al.[11]). The dose–response relationship for Bayrepel differs significantly from the other two.

testing but not in an olfactometer.[25] This is why we previously stated that K & D modules should be used only with chemicals having vapor pressures that are generally equivalent to that of deet. No system for testing mosquitoes has been shown to be definitive, in the sense of exactly mimicking what is found in field tests, which themselves are quite variable. As an example of the variability one finds in field tests, Traub and Elisberg,[26] in their Table 2, provide statistical summaries, where mean attack rates and their standard deviations are approximately the same for deet. Since mean attack rates have a hard lower limit of zero, this suggests that these data are strongly right skewed (highly variable). For the less protective combined insect repellent, M-2020 (also in their Table 2), mean attack rates and their variances are approximately the same (suggesting something similar to a Poisson process as a reasonable underlying model), again indicating high variability. However, the K & D module system is efficient and results are in basic agreement with other testing methods including field tests.

USE OF MODULES SIMILAR TO THE IN VITRO K & D MODULE BY OTHER RESEARCHERS

Tests of mosquitoes against various compounds of interest using a module system have also been conducted by Weldon et al.,[27,28] with a slightly different methodology, though using modules similar to those described above (the base had circular rather than square openings; the membranes used

were laboratory-made silicone wafers). The modules were placed over feeding wells, as described for the in vitro method above. Tests were run for 5 minutes. At the end of each minute, the behaviors of all (either five or six) mosquitoes in each cell in the module were recorded. Mosquitoes were categorized (three mutually exclusive categories) as either resting on the membrane (potentially feeding), flying, or resting on one of the other five surfaces of the cube. At the conclusion of the test, the module was closed and placed into a freezer to kill the mosquitoes, from which they were subsequently removed and squashed on a paper towel to determine if they had fed. If the feeding solution was clear (e.g., a sugar solution), green dye was added to it to make obvious those mosquitoes that fed. Additional details are given in the original articles cited earlier.[27,28]

The K & D module system was modified in a different way by Zhu[29,30] for use with stable flies, *Stomoxys calcitrans* (Linnaeus) and house flies, *Musca domestica* (Linnaeus). Three to five flies were placed in each cell of the module. Rather than using wells containing liquid, squares cut from a feminine hygiene pad were soaked either in citrated bovine blood for stable flies or in a red-dyed sugar solution for house flies, topically coated with the compound of interest, and were placed in the module wells. Tests were run for 4 hours. The dependent variables measured were whether individual flies fed (assayed by squashing the abdomens of the flies), time to knock down (flies lying on the floor of the box, unable to fly and abdomen up), and time to death (flies considered dead did not move when prodded with a thin wooden stick).

STATISTICAL ANALYSIS OF DATA FROM THE K & D AND SIMILAR MODULES

There are several useful kinds of data one can collect using this module system when testing compounds. The usual measure is whether or not a mosquito has ingested the feeding solution through the membrane. This can be determined visually (mosquito has mouthparts inserted through the membrane and the abdomen is distended and red), or the mosquitoes can be squashed at the test's conclusion. Blood feeding is easy to see, but food coloring dye should be added to colorless feeding solutions. Other behavioral measures can be useful, for example, one can census the mosquitoes in each cell at a given time point (or at several time points), and note the behavior of each mosquito (typically categorized as resting on the membrane, flying, or resting on one of the other five surfaces of the cell).[26,27]

Comparing Compounds and Concentrations Using Feeding

We first discuss the analysis of feeding counts, subsequently the analyses of other behaviors, how all behaviors observed can be combined into a composite score, and random effects useful in statistical modeling of these kinds of data.

To statistically model whether mosquitoes have fed, visually or using the squashed mosquito assay, we assume the mosquitoes are samples from a binomial distribution. That is, individual mosquitoes fed or not fed, and these counts are summed over the cell or cells. The binomial parameter, which models the proportion of feeding mosquitoes, depends in large part on how the chemical applied to the membrane affects feeding. If no chemical is applied (the control condition), most mosquitoes should feed. As chemicals become more effective, or are applied at higher concentrations, fewer mosquitoes should feed. There are other potential variables affecting the proportion of feeding mosquitoes, these include time of day, room environment (temperature, relative humidity), and mosquito characteristics (e.g., age, species).

The binomial distribution, unlike the normal distribution, has only one parameter, which, in conjunction with the known sample size (sample size is a constant, it is not estimated), determines both the mean and variance. The normal distribution has two independent parameters, one for the mean and one for the variance. The traditional way to handle binomial data was to use a variance

stabilizing formula, for example, $\sin^{-1}\sqrt{y/n}$, where y is the number of feeding mosquitoes (an equivalent analysis defines y as the proportion of nonfeeding mosquitoes) and n is the total number of mosquitoes. An improved formula is $\sin^{-1}\sqrt{(y+3/8)/(n+3/4)}$. One then applies statistical tests for normally distributed data (e.g., t-test, ANOVA). With modern statistical software, use of these transformations is no longer necessary. In fact, the transformations do not work well when y/n is small, as occurs when the chemicals tested are effective, nor when y/n is near 1.0, for example, for controls. The current preferred method is to use software that estimates a generalized linear model[31] using the logit link for binomial data, sometimes called logistic regression. Some prefer the probit to the logit link, the differences between them are small. Essentially, instead of modeling the proportion directly, one models the expected value of the logit of the proportion, $p = y/n$, that is, E [log $(p/\{1 - p\})$], where the logit (p) (the dependent variable) is influenced by chemical, concentration, environment, and mosquito characteristics (the independent variables), and E is the expectation function. These models have better statistical properties for modeling binomial data than using transformations. Statistical tests for differences between chemicals will be more accurate, other independent variables can easily be added to the models in a regression-like manner to look at their influence, the change in the variance, as a function of the mean, is correctly accounted for, and confidence intervals will make sense. When using a transformation, it is quite possible to have a 95% confidence interval on a proportion that includes negative numbers.

However, analyzing data in this framework reveals a weakness of basing a statistical model on the binomial distribution because biological data does not usually conform to theory. In particular, for binomial data, there is usually more variability than expected from a pure binomial process, even taking into consideration the other independent variables mentioned earlier. This unaccounted for additional variability is known as over-dispersion. The fix is to add an over-dispersion parameter to the model, which corrects the often too small standard errors and make appropriate adjustments on statistical tests. An alternative fix is to include random effects at the individual level, for example, for each cell, although not all software will allow this. Another problem occurs if there are compounds that were 100% repellent, where no mosquitoes fed at that compound/concentration combination. Including these data in the analysis without adjustment leads to problems, typically by producing enormous standard errors in the output (when actually, at a proportion of 0% or 100%, the variance is zero). There are two remedies: either do not include the data in the analysis, which makes sense because if the variance is zero, there will be a significant difference between this compound and any other, or change one of the zero bites to a fraction (0.1); even though binomial data consists of counts, putting in a fraction rather than an integer does not break the software, though it may produce a warning message. However, any comparisons made involving this compound are suspect because the data have been altered.

In the framework of a generalized linear model, linear model decompositions (contrasts) are done in the usual traditional way. For example, if one compares controls against each of the test compounds, this is an a priori linear contrast and sufficient degrees of freedom exist to avoid a multiple comparisons adjustment. However, if one also compares each compound to every other one, or against a deet positive control, then one is making more comparisons than allowed for with the degrees of freedom (the multiple comparisons scenario) and an adjustment, either on the test statistic or the p value, is needed. A Bonferroni adjustment is an example of an adjustment on the p value; better methods exist—a contemporary one is to adjust for the false discovery rate.

Often a compound is tested at several concentrations with the aim of constructing a "dose–response" curve. We suggest use of the generalized linear model framework. The key is to find an appropriate transformation of the concentration so that the relationship between the logit (or probit) of the proportion of bites and concentration is a straight line. If there is only one compound involved, this is usually not difficult, but sometimes a transformation on dose creates a straight line relationship for one compound but not for others, which is problematic because the goal is to

compare linear slopes. In that case, the slopes can only be compared in regions of concentration where the same transformation can be used for all compounds. An alternative is to fit a polynomial (linear and quadratic components) to the transformed concentration, and compare and interpret both components. In our work, we have transformed concentration using the log, square root, and identity (no transformation).

Some compounds seem to be completely ineffective at low doses, but then exhibit a normal dose–response curve at higher concentrations. In this case, we have modeled the dose–response curve as flat, that is, no change from control, until some low subjectively estimated concentration, then rising from that point (Picaridin in Figure 14.7). In the statistical model, one simply subtracts this low estimated concentration from all the others so it becomes the new "zero" and then estimates the model in the usual way. For high concentrations, where no mosquitoes feed, the same reasoning applies as discussed earlier, either those concentrations should be dropped from the analysis or the data need to be slightly altered to allow the curve to be fit. However, in this case, one can choose how to alter the data such that this final high concentration does not affect the model fit (the altered point sits close to the line fitted without the point). Neither of these alternatives for fixing compound/concentration combinations of zero is satisfying. In theory, the zero proportion estimate results from sampling error. That is, if sufficient numbers of mosquitoes were tested, at least one would have fed, so the true value at this concentration is nonzero.

Including controls, where no compound is applied to the membrane, provides both a measure of experiment-to-experiment consistency and a baseline against which to measure the activity of compounds of interest. Kramer et al.[32] investigated the statistical properties of testing mosquitoes in modules and found that the correlation between control biting rates and treatment biting rates on deet-exposed membranes in the same trial was 0.67 ($p = .002$). It is unclear how to interpret this number. Although there is clearly a strong relationship between the two, it is not strong enough for the control values to be used to adjust the deet values of different experiments to a common level. There were even trials where bites by mosquitoes in the deet treatment approached or exceeded those by controls. Further, they found that, ignoring sampling error, most (61%) of the variation among controls was not accounted for (i.e., was not due to day-to-day or session-to-session variability), indicating that analyses for these kinds of data need to allow for over-dispersion, discussed earlier. These results also suggest that large sample sizes be used, because that will allow for a good estimate of the additional unaccounted for uncertainties as an over-dispersion parameter. Luckily, for mosquito researchers, large samples sizes are easy to obtain.

Comparing Compounds When Additional Behaviors Have Been Recorded

The proportion of landing or flying is analyzed in a different way if mosquitoes are repeatedly measured, that is, every minute in a 5-minute test, as done by Weldon et al.[27] Mosquitoes at each observation period were classified into one of three categories, landing on the membrane, landing elsewhere, and flying, for example, Weldon et al.,[27,28] but only two of the three categories were analyzed because the sum of the counts in all categories is always the product of the number of mosquitoes in the cell and the number of times measurements were made, and the tests are then not independent. Because one is repeatedly measuring the same unmarked individuals and then summing all the counts, the data are no longer strictly binomial, which assumes the counts are independent. Given that, in a 5-minute test, one could have a maximum count of 25, one can apply the standard (or improved, given earlier) variance stabilizing transformation for proportions, on count/25. The transformed proportions can then be analyzed using a statistical model for normally distributed data, for example, ANOVA. This was the approach taken by Weldon et al.[27,28]

If there are two or more concurrently measured behaviors on each group of five mosquitoes, for example, landing on the membrane, flying, and feeding, an alternative that we have found works well

to rank a large number of compounds tested concurrently is to use the methods given in Kramer et al.,[33] where a composite score is created (a single score for each cell of five mosquitoes based on optimal weightings of the concurrently measured behaviors) that maximizes the differences among compounds. This method was used for mosquitoes in Weldon et al.[25] If other variables, for example, time of day, had a large influence on the outcome of tests, those variables could also be included when creating the composite score. If the resulting scores are close to normally distributed, then the usual linear models can be used to test for differences. In Weldon et al.,[25] where distributions of the resulting scores were decidedly skewed, differences were tested using the a posteriori Kruskal–Wallis method.[34]

RANDOM EFFECTS

In general, any time humans or other vertebrates are used as potential hosts in an experiment, there are individual differences in their attractiveness to mosquitoes, and this source of variation should be included in the statistical model. These are random effects because, if the experiment was run again, likely a different set of potential hosts would be used (versus a fixed effect like concentration or time of day). Another potential random effect that may need to be included in the statistical model is a block effect, for example, if the experiment was repeated over a few days, there may be a random day effect. If random effects are included in the model, then the model framework changes to linear mixed models or generalized linear mixed models. Current statistical software can estimate these models and they should be used because they more accurately reflect the process producing the data and thus give better statistical tests. However, since the software is relatively new, it is also less mature, and estimation problems are more likely, especially if there are compound/concentration combinations with no mosquitoes feeding, as mentioned earlier. Also, model diagnostics are not as far along, though the situation should improve with time.

CONCLUSIONS

K & D Module Use

The K & D module system was developed and overcame problems in previously used module systems. By creating cells containing only a few mosquitoes, a larger number of compounds can be concurrently tested, which is both a better statistical design that allows for higher throughput when screening, and uses fewer mosquitoes. Even higher throughput can be achieved by eliminating human volunteers and testing mosquitoes in an in vitro system, where they can feed through a membrane under which lies wells of a blood substitute, kept warm using circulating water. This methodology has been adopted by other researchers, demonstrating the usefulness of the system.

Data Analysis

If only one dependent variable (feeding) is observed, data analysis is straightforward, although over-dispersion of the data (relative to a binomial distribution) is typically present and needs to be taken into account in the analysis. If more than one dependent variable is observed, the composite score technique[33] is effective for reducing the dimensionality of the data. Random effects are also typical of the experimental designs used, such as those resulting from different volunteers in in vivo trials, day-to-day differences, and so on and should not be ignored because doing so makes for too liberal tests (p values are too small). They can be included if the data are analyzed in the generalized linear mixed model framework.

REFERENCES

1. J. A. Klun and M. Debboun, A new module for quantitative evaluation of repellent efficacy using human subjects, *J. Med. Entomol.*, 37: 177–181, 2000.
2. R. E. Coleman, L. L. Robert, L. W. Roberts, J. A. Glass, D. C. Seeley, A. Laughinghouse, P. V. Perkins, and R. A. Wirtz, Laboratory evaluation of repellents against four anopheline mosquitoes (Diptera: Culicidae) and two phlebotomine sand flies (Diptera: Psychodidae), *J. Med. Entomol.*, 30: 499–502, 1993.
3. American Society for Testing and Materials, Standard test methods for laboratory testing of non-commercial mosquito repellent formulations on the skin. Standard E951-83. American Society for Testing and Materials, Philadelphia, PA, 1983.
4. M. D. Buescher, L. C. Rutledge, R. A. Wirtz, K. B. Glackin, and M. A. Moussa, Laboratory tests of repellents against *Lutzomyia longipalpis* (Diptera: Psychodidae), *J. Med. Entomol.*, 19: 176–180, 1982.
5. R. K. Gupta, L. C. Rutledge, and W. J. Letourneau, An improved laboratory test cage for testing repellents on human volunteers, *J. Amer. Mosq. Control Assoc.*, 5: 436–438, 1989.
6. L. C. Rutledge and R. K. Gupta, Evaluation of an in vitro blood feeding station for testing mosquito repellents, *J. Amer. Mosq. Control Assoc.*, 20: 150–154, 2004.
7. G. Casella, *Statistical Design*, Springer, New York, 2008.
8. J. A. Klun, D. Ma, and R. Gupta, Optically active arthropod repellents for use against disease vectors, *J. Med. Entomol.*, 37: 182–186, 2000.
9. J. A. Klun, W. Schmidt, and M. Debboun, Stereochemical effects in an insect repellent, *J. Med. Entomol.*, 38: 809–812, 2001.
10. J. A. Klun and W. Schmidt, Methods and compositions for repelling arthropods, S. N. 09/978,154, D. N. 0091.00, Patent Issued 5/13/2003.
11. J. A. Klun, A. Khrimian, A. Margaryan, M. Kramer, and M. Debboun, Synthesis and repellent efficacy of a new chiral piperidine analog: Comparison with Deet and Bayrepel activity in human-volunteer laboratory assays against *Aedes aegypti* and *Anopheles stephensi, J. Med. Entomol.*, 40: 293–299, 2003.
12. J. A. Klun, A. Khrimian, and M. Debboun, Repellent and deterrent effects of SS220, Picaridin, and Deet suppress human blood feeding by *Aedes aegypti, Anopheles stephensi*, and *Phlebotomus papatasi, J. Med. Entomol.*, 43: 34–39, 2006.
13. V. G. Dethier, L. B. Browne, and C. N. Smith, The designation of chemicals in terms of the responses they elicit from insects, *J. Econ. Entomol.*, 53: 134–136, 1960.
14. J. A. Klun, M. Kramer, and M. Debboun, A new in vitro bioassay system for discovery of novel human-use mosquito repellents, *J. Amer. Mosq. Control Assoc.*, 21: 64–70, 2005.
15. AABB, *Technical Manual* 15th Edition, American Association of Blood Banks, Bethesda, MD, 2005.
16. J. A. Klun, M. Kramer, A. Zhang, S. Wang, and M. Debboun, A quantitative in vitro assay for chemical mosquito-deterrent activity without human blood cells, *J. Amer. Mosq. Control Assoc.*, 24: 508–512, 2008.
17. C. L. Cantrell, J. A. Klun, C. T. Bryson, M. Kobaisy, and S. O. Duke, Isolation and identification of mosquito bite deterrent terpenoids from leaves of American (*Callicarpa americana*) and Japanese (*Callicarpa japonica*) beautyberry, *J. Agric. Food Chem.*, 53: 5948–5953, 2005.
18. J. F. Carroll, C. L. Cantrell, J. A. Klun, and M. Kramer, Repellency of two terpenoid compounds isolated from *Callicarpa americana* (Lamiaceae) against *Ixodes scapularis* and *Amblyomma americanum* ticks, *Exp. Appl. Acarol.*, 41: 215–224, 2007.
19. A. Zhang, J. A. Klun, S. Wang, J. F. Carroll, and M. Debboun, Isolongifolenone: A novel sesquiterpene repellent of ticks and mosquitoes, *J. Med. Entomol.*, 46: 100–106, 2009.
20. D. E. Wedge, J. A. Klun, N. Tabanca, B. Demirci, T. Ozek, K. H. C. Baser, Z. Liu, S. Zhang, C. L. Cantrell, and J. Zhang, Bioactivity-guided fractionation and GC/MS fingerprinting of *Angelica sinensis* and *Angelica archangelica* root components for antifungal and mosquito deterrent activity, *J. Agric. Food Chem.*, 57: 464–470, 2009.
21. C. L. Cantrell, A. Ali, S. O. Duke, and I. Khan, Identification of mosquito biting deterrent constituents from the Indian folk remedy plant *Jatropha curcas, J. Med. Entomol.*, 48: 836–845, 2011.
22. A. M. P. Jones, J. A. Klun, C. L. Cantrell, D. Ragone, K. R. Chauhan, P. N. Brown, and S. J. Murch, Isolation and identification of mosquito (*Aedes aegypti*) biting deterrent fatty acids from male inflorescences of breadfruit (*Artocarpus altilis* (Parkinson) Fosberg), *J. Agric. Food Chem.*, 60: 3867–3873, 2012.

23. A. Ali, C. L. Cantrell, U. R. Bernier, S. O. Duke, J. C. Schneider, N. M Agramonte, and I. Khan, *Aedes aegypti* (Diptera: Culicidae) biting deterrence: Structure-activity relationship of saturated and unsaturated fatty acids, *J. Med. Entomol.*, 49: 1370–1378, 2012.
24. A. Zhang, J. F. Carroll, S. Wang, and J. A. Klun, Methods for repelling arthropods using isolongifolenone analogs, U.S. Patent 7,579,016, 2009.
25. P. J. Weldon, J. F. Carroll, M. Kramer, R. H. Bedoukian, R. E. Coleman, and U. R. Bernier, Anointing chemicals and hematophagous arthropods: Responses by ticks and mosquitoes to citrus (Rutaceae) peel exudates and monoterpene components, *J. Chem. Ecol.*, 37: 348–359, 2011.
26. R. Traub and B. L. Elisberg, Field tests on diethyltoluamide (Deet), a highly effective repellent against mosquitoes in the Nipah palm-mangrove swamps in Malaya, *Pac. Insects*, 4: 303–313, 1962.
27. P. J. Weldon, J. R. Aldrich, J. A. Klun, J. E. Oliver, and M. Debboun, Benzoquinones from millipedes deter mosquitoes and elicit self-anointing in capuchin monkeys (*Cebus spp.*), *Naturwissenschaften*, 90: 301–304, 2003.
28. P. J. Weldon, M. Kramer, S. Gordon, T. F. Spande, and J. W. Daly, A common pumiliotoxin from poison frogs exhibits enantioselective toxicity against mosquitoes, *Proc. Natl. Acad. Sci.*, 103: 17818–17821, 2006.
29. J. J. Zhu, X. P. Zeng, D. Berkebile, H.J. Du, Y. Tong, and K. Qian, Efficacy and safety of catnip (*Nepeta cataria*) as a novel filth fly repellent, *Med. Vet. Entomol.*, 23: 209–216, 2009.
30. J. J. Zhu, A. Y. Li, S. Pritchard, K. Tangtrakulwanich, F. P. Baxendale, and G. Brewer, Contact and fumigant toxicity of a botanical-based feeding deterrent of the stable fly, *Stomoxys calcitrans* (Diptera: Muscidae), *J. Agric. Food Chem.*, 59: 10394–10400, 2011.
31. P. McCullagh and J. A. Nelder, *Generalized Linear Models* 2nd Edition, Chapman and Hall, New York, 1989.
32. M. Kramer, M. F. Feldlaufer, and K. R. Chauhan, Mosquito biting behavior: Statistical power and sources of variation in toxicity and repellent bioassays, *J. Med. Entomol.*, 47: 199–204, 2010.
33. M. Kramer, P. J. Weldon, and J. F. Carroll, Composite scores for concurrent behaviours constructed using canonical discriminant analysis, *J. Animal Behav.*, 77: 763–768, 2009.
34. W. H. Kruskal and W. A. Wallis. Use of ranks in one-criterion variance analysis, *J. Amer. Stat. Assoc.*, 47: 583–621, 1952.